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14. ABSTRACT The effects of soy on cancer metastasis remain to be understood. A recent study by our laboratory reported that the soy isoflavone genistein reduced tumor growth and metastasis and downregulated cancer promoting genes in a nude mouse model with tumors established from MDA-MB-435 metastatic cancer cells. On the contrary individual daidzein and combined isoflavones (genistein, daidzein, glycitein) increased metastasis and upregulated the expression of genes that promote cell proliferation and survival, including eukaryotic protein synthesis initiation factors *EIF4GI* and *EIF4E,* critical members of the eIF4F protein synthesis initiation factor complex. The hypothesis of the proposed research is that soy isoflavones modulate breast cancer progression by specific regulation of eIF4F complex to affect the synthesis of cancer regulatory proteins. The purpose of this study is to investigate the molecular mechanisms by which soy isoflavones genistein and daidzein disparately regulate protein synthesis initiation in established breast cancer. Herein, we show that individual or combined soy isoflavones genistein, daidzein, and glycitein at physiological concentrations in MDA-MB-231 and SKBR3 did not change eIF4F complex or mTOR pathway. Treatment of MDA-MB-435 cells with combined soy isoflavones increased eIF4E protein expression. Results with MDA-MB-231 and MDA-MB-435 metastatic cancer cell lines show that equol, but not daidzein, upregulated eIF4G without affecting eIF4E or its regulator 4E binding protein (4E-BP) levels. Equol also increased c-Myc levels, as well as expression of IRES containing cell survival and proliferation promoting molecules. Daidzein metabolite equol may be a potent regulator of the cancer promoting effects of dietary daidzein.

15. SUBJECT TERMS

Key Words: breast cancer, metastasis, soy isoflavones, genistein, daidzein, glycitein, equol, protein synthesis initiation, eIF4G, eIF4E, 4E-BP, mTOR, cap-independent, IRES.

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Title: "Role of Protein Synthesis Initiation Factors in Dietary Soy Isoflavone-Mediated Effects on Breast Cancer Progression"

INTRODUCTION

Isoflavones found primary in legumes, particularly in soy, are a major class of phytoestrogens which are structurally and/or functionally similar to 17β estradiol (1). Since soy foods have anti cancer effects at early stages of carcinogenesis, most studies have focused their investigation on prevention of breast cancer risk using soy isoflavones (1;2). However, the benefits of soy foods as chemopreventives for established breast cancer or as substitutes for hormone replacement therapies remain controversial (3). A recent study by our laboratory to investigate the role of dietary soy isoflavones in established breast cancer, reported that the soy isoflavone genistein reduced tumor growth and metastasis and downregulated cancer promoting genes in a nude mouse model of experimental metastasis (4). On the contrary, individual daidzein and combined isoflavones (genistein:daidzein:glycitein, 5:4:1) increased metastasis and upregulated the expression of genes that promote cell proliferation and survival, including eukaryotic protein synthesis initiation factors *EIF4GI* and *EIF4E,* critical members of the eIF4F protein synthesis initiation factor complex. Therefore **the hypothesis** of the proposed research is that soy isoflavones modulate breast cancer progression by specific regulation of eIF4F complex to affect the synthesis of cancer regulatory proteins. The **purpose** of this study is to investigate the molecular mechanisms by which soy isoflavones genistein and daidzein disparately regulate protein synthesis initiation in established breast cancer.

BODY

<u>Progress towards the proposed research objectives/Specific Aims</u>:

Specific Aim 1*: *Delineate the effects of genistein, daidzein, glycitein or combined soy isoflavones on mammalian target of rapamycin (mTOR) signaling.

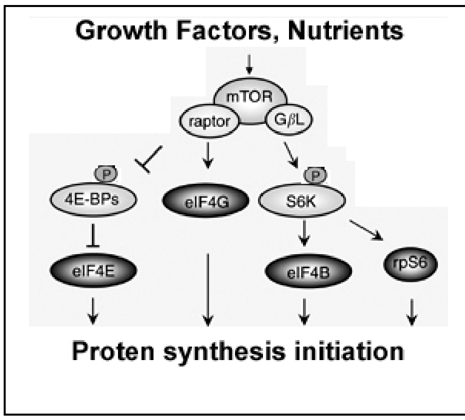

Figure 1. mTOR activity. Activation of mTOR by growth factors and nutrients results in mTOR-mediated phosphorylation of two targets: 4E binding protein (4E-BP) and p70S6 kinase (S6K). Phosphorylation of 4E-BP results in release of eIF4E to initiate cap-dependent protein synthesis with eIF4G. Phosphorylation and activation of S6K also results in ribosome biogenesis and protein synthesis initiation via phosphorylation of ribosomal S6 subunit (rpS6) and eIF4B.

The objective of Aim 1 was to determine whether soy isoflavones affect the activity and expression of eIF4F initiation factors by determining changes to total and phospho (active) forms of the mTOR signaling pathway (Fig. 1).

Effect of soy isoflavones (genistein, daidzein, and glycitein) on expression of protein synthesis initiation factors and mTOR effector 4E-BP:

Since our preliminary data demonstrated increased eIF4E and eIF4G gene and protein expression in response to dietary daidzein or combined soy isoflavones, we focused first, on elucidating the most effective concentration or combination of soy isoflavones that better modulate members of the eIF4F complex: eIF4G and eIF4E. Genistein, daidzein, and glycitein and their respective glycosides account for ~50%, 40%, and 10%, respectively, of total soybean isoflavone content (5;6). Therefore, our study was conducted with genistein:daidzein:glycitein in the ratio of 5:4:1 to mimic the composition of soy isoflavones consumed by humans, and at physiological concentrations that accumulate in the circulation following consumption of soy foods (5-7). Metastatic human breast cancer cells were treated with 20 μM, 16 μM, 4 μM of genistein, daidzein and glycitein, individually or in combination. In MDA-MB-231 (ERα-, ERβ+, EGFR+), and SKBR3 (ER-, EGFR+, HER2++) cell lines, individual or combined soy isoflavones did not significantly change the expression of the eIF4F family members tested, or the mTOR target, the inhibitor of eIF-4E, 4E-BP1 phosphorylation (Fig. 2). This result indicates that neither eIF4F expression nor mTOR signaling is affected by soy isoflavones at these concentrations, in low metastatic human breast cancer cells.

The hypothesis that soy isoflavones modulate breast cancer progression by specific regulation of the eIF4F eukaryotic initiation factor complex members was based on a previous study from our laboratory that reported that dietary administration of daidzein or soy isoflavones increased mammary tumor growth and metastasis of a ER (-) highly metastatic human cancer cell line MDA-MB-435 *in vivo* (4). Since soy isoflavone treatment did not change eIF4F or the mTOR target 4E-BP expression or activity in the low metastatic cancer cell lines MDA-MB 231 and SKBR3, we decided to test the effect of soy isoflavones using the same cell line from our *in vivo* study, the highly metastatic ER (-) HER2 ++ MDA-MB-435 cells. Similar to our results *in vivo*, where eIF4E was the only gene in the PI3-K pathway PCR array that was upregulated by combined soy isoflavones, the combined soy isoflavones increased eIF4E protein levels by ~1.7-fold *in vitro*. However, none of the individual isoflavones exerted a significant effect on eIF4F expression (Fig. 3).

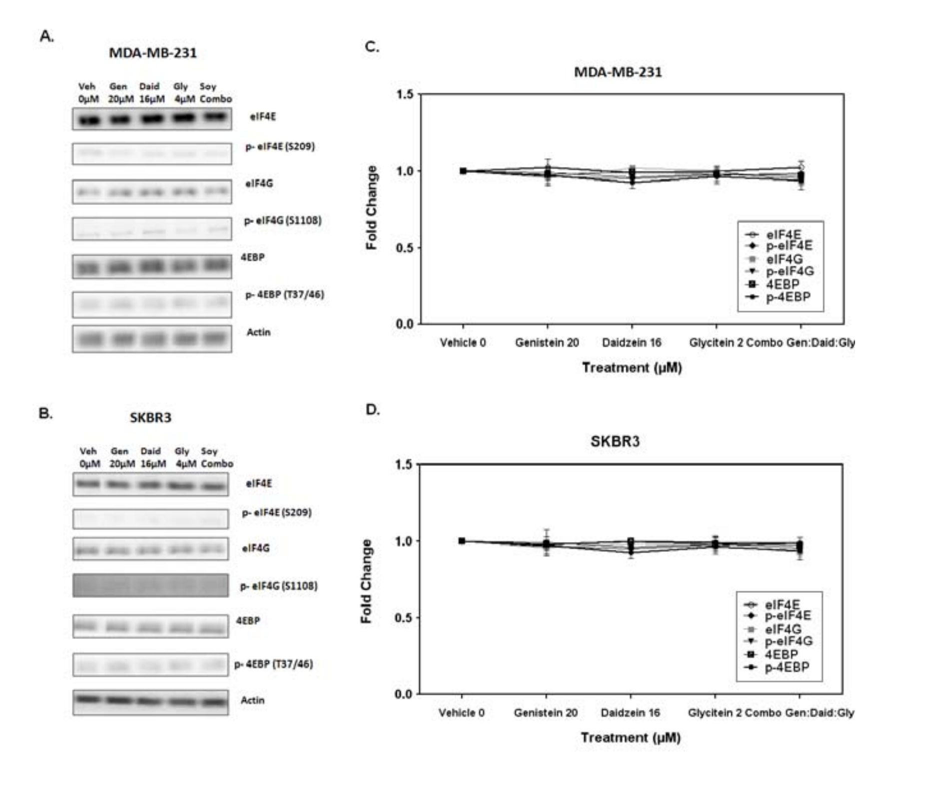

Figure 2. Effect of soy isoflavones: genistein (Gen), daidzein (Daid), or glycitein on protein expression of breast cancer cells. MDA-MB-231 (**A,C**) or SKBR3 (**B,D**) cells were treated for 24 h with individual or combined genistein (20 μM), daidzein (16μM), and glycitein (4 μM) and western blotted for total or phospho-forms of initiation factors and 4E-BP. **A,B,** Representative western blots. **C,D,** Fold changes compared to vehicle as calculated from the integrated density of positive bands from western blots and normalized with actin expression. N=2±SD.

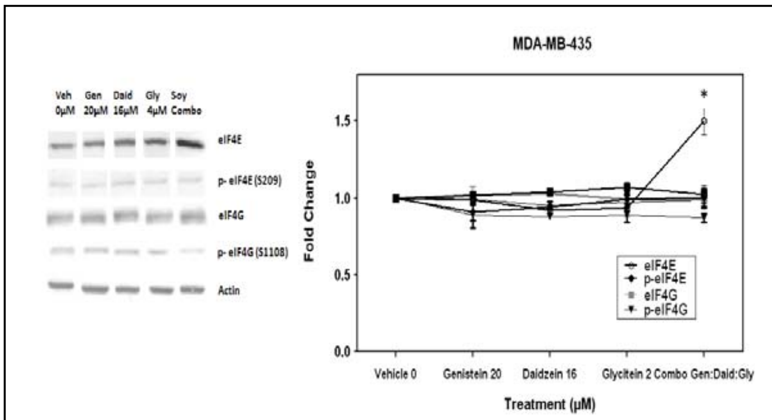

Figure 3. Effect of soy isoflavones on total and phospho (p) eIF4E and eIF4G expression in MDA-MB 435 cells. Quiescent MDA-MB-435 cells were treated for 24 h with vehicle (0.1% DMSO), 20 μM genistein, 16 μM daidzein, 4 μM glycitein, or genistein, daidzein, and glycitein in the same concentrations. Representative western blots and fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of MDA-MB 435 cell extracts. Values show mean ± SEM (N=3). An asterisk indicates statistical significance of p<0.05.

We are cognizant of the controversy surrounding the ***MDA-MB-435 cell line***. The origin of the MDA-MB-435 cell line has been questioned by microarray studies and is considered to be a melanoma (8). However, as recently reviewed in (9), these cells may still be of breast cancer origin but showing aberrant lineage infidelity, a wide spread phenomenon in cancer (10). Similar to MDA-MB-435, melanocyte-related genes have been found in human breast cancer specimens (11). Therefore, we have continued the use of this cell line as a model system for a highly metastatic HER2 type breast cancer, but we would like to emphasize that all of the results for this proposal will be confirmed with MDA-MB-231 and MDA-MB-468 cells.

Since the *in vivo* effect of daidzein on eIF4G and eIF4E expression was not simulated *in vitro* with 16 μM daidzein, a dose response curve was conducted to determine if the reason we could not recapitulate the *in vivo* results was concentration-dependent. MDA-MB-435 and MDA-MB-231 cells were treated with vehicle or daidzein at concentrations ranging from 0-50 μM for 24h. Figure 4 shows representative western blots of

MDA-MB-231 and MDA-MB-435 cells following daidzein treatment that demonstrate no significant change in total or phospho- eIF4E, eIF4G, or 4E-BP. The slight increases in eIF4G and phospho-eIF4G in MDA-MB-231 cells following 25 and 50 μM daidzein were not statistically significant (p>0.05).

Effect of equol on expression of protein synthesis initiation factors and mTOR effector 4E-BP:

Daidzein can be further metabolized to equol (70%) and O-desmethylangolensin (O-DMA) (5-20%), *in vivo* by gut bacteria (12). Equol has 80 times more ERβ affinity and higher bioavailability than daidzein.

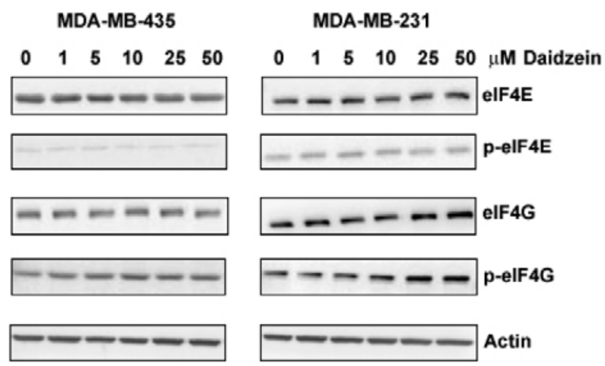

Figure 4. Effect of daidzein on total and phospho (p) eIF4E and eIF4G expression in MDA-MB-435 and MDA-MB 231 cells. Quiescent cells were treated with vehicle or daidzein (0-50 μM) in 5% serum for 24 h, lysed, and western blotted with mono-specific antibodies. Left, representative western blots of MDA-MB 435 cell lysates. Right, representative western blots of MDA-MB 231 cell lysates. N=3.

Not all humans have the gut microflora necessary to convert daidzein to equol. Approximately 30–50% of humans are equol producers. In rodents, equol is the major circulating metabolite and all rodents are equol producers (12;13). Therefore, we reasoned that the daidzein effects may be due to the metabolite equol. Thus, the effect of the daidzein metabolite (R,S)-equol was tested in MDA-MB-231 and MDA-MB-435 cells at varying concentrations (0-50 μM).

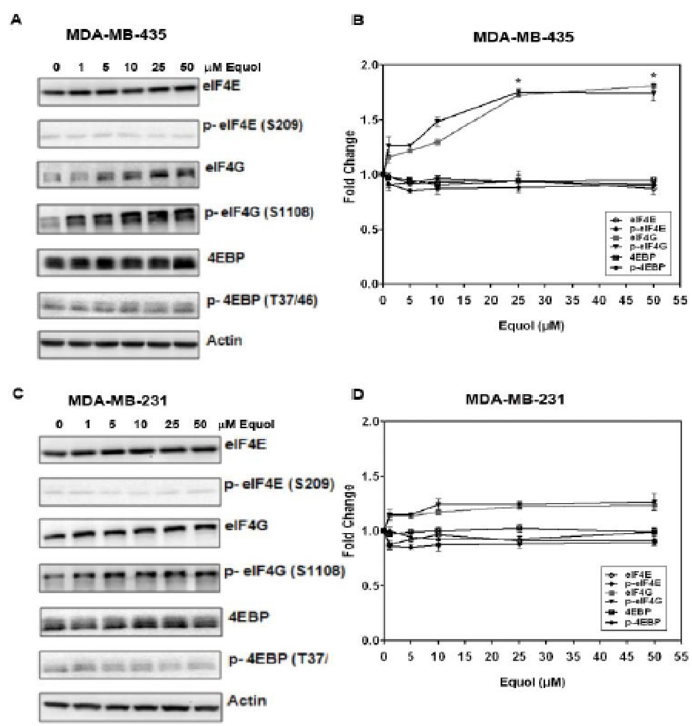

Figure 5. Effect of equol on total and phospho (p) eIF4E, eIF4G, and 4E-BP expression in MDA-MB 435 and MDA-MB 231 cells. Quiescent cells were treated with vehicle or equol (0-50 μM) for 24 h, lysed, and western blotted with mono-specific antibodies. (A,B) Representative western blots and fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of MDA-MB 435 cell extracts. (C,D) Representative western blots and fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of MDA-MB 231 cell extracts. Values show mean ± SEM (N=3). An asterisk indicates statistical significance of *p*<0.05.

Figure 5 demonstrates that the metabolite equol increases the expression of total and phospho eIF4G in a concentration-dependent manner by ~1.8-fold at 25 μM and 50 μM (p<0.05) in MDA-MB-435 cells. MDA-MB-231 cells demonstrated a similar trend by increased eIF4G expression in response to equol. The protein levels of eIF4E and 4E-BP remained unchanged in both cell lines, suggesting that the mTOR signaling pathway was not affected. However, equol or combined isoflavones with equol (genistein:equol:glycitein) did not change eIF expression in SKBR3 cells (Fig.6). Currently, we do not have a logical explanation for this result, except that SKBR3 cells express a number of EGFR isoforms in addition to HER2 and genistein may act as a tyrosine kinase inhibitor in this cell line, thus, inhibiting signaling from EGFR to regulate expression of cancer promoting molecules. Future studies will determine the effect of equol on eIF4G expression and protein synthesis in a range of breast cancer cell lines, representing a range of breast cancer subtypes (i.e. luminal A, luminal B, triple negative/ basal like, and HER2 type) determine if this effect is cell type dependent.

Conclusions from Aim 1: Aim 1 demonstrated that total and phospho- eIF4E and eIF4G levels were increased by combined soy isoflavones and equol respectively. Since our PCR array data from tumors showed a similar increase in gene expression, we conclude that increased eIF4E and eIF4G by soy isoflavones is

regulated at the level of gene expression.

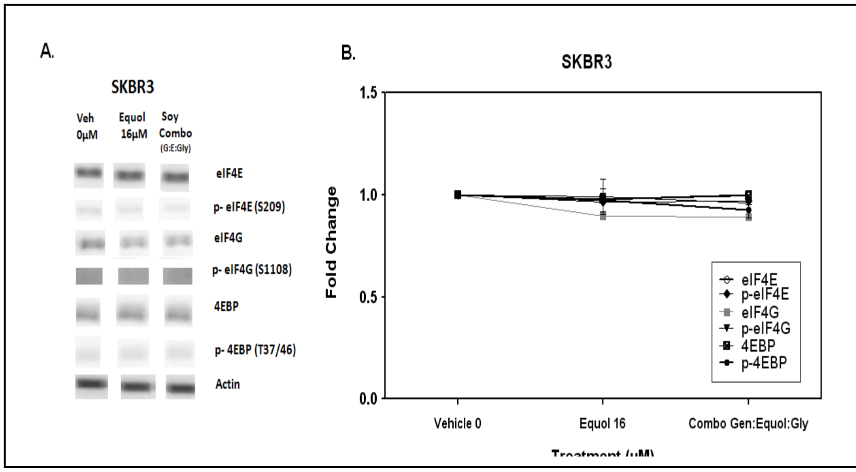

Figure 6. Effect of equol on total and phospho (p) eIF4E, eIF4G, and 4E-BP expression in SKBR3 cells. Quiescent cells were treated with vehicle or daidzein (0-50 μM) in 5% serum for 24 h, lysed, and western blotted with mono-specific antibodies. Representative western blots and fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of SKBR3 cell extracts. Values show mean ± SEM (N=2).

Since none of the compounds, in the three cell lines tested, changed the phosphorylation status of 4E-BP, which is a direct target of mTOR kinase, we conclude that ***soy isoflavones do not regulate mTOR signaling***.

However, since eIF4E and eIF4G levels are increased by soy isoflavones, we are continuing to test the original ***hypothesis that soy isoflavones modulate breast cancer progression by specific regulation of the eIF4F eukaryotic initiation factor complex to affect the synthesis of cancer regulatory proteins***.

Specific Aim 2: ***Investigate the effects of genistein, daidzein, glycitein or combined soy isoflavones on expression of mRNAs that are specifically regulated by the eIF4F initiation complex.***

This Aim has been initiated by investigation of the expression of pro-cancer molecules with internal ribosomal entry sites (IRES) containing mRNAs that were elevated at the protein level in the HER2 ++ ER (-) MDA-MB-435 cells.

Effect of equol on pro-cancer molecules sensitive to eIF4F levels:

To investigate the effect of the overexpressed eIF4F complex on translation of mRNAs sensitive to elevated eIF4F initiation factors, we analyzed protein expression levels of molecules that regulate cancer cell survival and proliferation in MDA-MB435 cell lines followed by equol treatments. Translation process has a crucial role in cancer development. The eIF4F family initiation factors have been shown to be overexpressed in advanced cancer and to be essential for translation of a subset of proteins that regulate cellular bioenergetics, survival, and proliferation (14-16). The canonical process of translation implies recognition of the cap structure (7-methyl-guanosine) at the 5' end of the mRNA by eIF4E. Association of eIF4E with the cap permits binding of a scaffolding protein, eIF4G, bringing the cap to the remainder of the translational machinery (14;15). An alternative and less understood mechanism of certain mRNAs, first discovered in the picornavirus family and now found in eukaryotic mRNAs, utilizes the 5' leader to recruit the translational machinery through an IRES. eIF4G is thought to bind directly and recruit 40S ribosome subunits and other factors to initiate protein synthesis (14-16). High levels of eukaryotic initiation factors, specifically eIF4GI have been correlated with increased translation of the cap independent mechanism of specific mRNAs that contain IRESs (17;18). These molecules have mRNAs with long UTRs and/or IRESs (14-

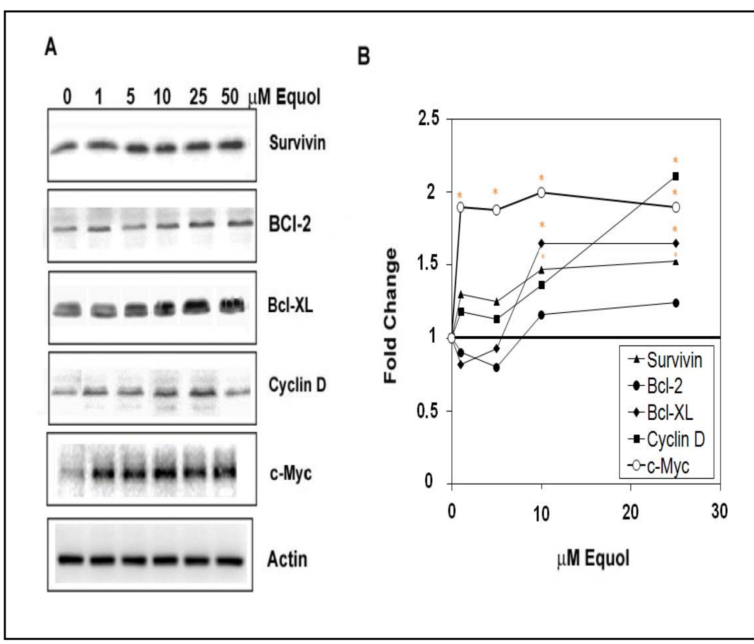

Figure 7. Expression of pro-cancer proteins following equol treatment. Quiescent MDA-MB-435 cells were treated with vehicle or 1-50 μM equol for 24 h, lysed, and western blotted with specific antibodies to survivin, Bcl-XL, Bcl2, Cyclin D and c-Myc. (A) Representative western blots. (B) Fold changes relative to actin were calculated by Image J analysis of positive bands from equol treatments (1-25 μM) compared to vehicle controls. N=3. An asterisk indicates statistical significance of $p<0.05$.

20). Figure 7 demonstrates that equol upregulates protein expression of such IRES containing pro-cancer molecules that regulate cancer cell proliferation and survival: c-myc, survivin, Bcl-2, Bcl-XL and Cyclin D, by ~1.3-1.7-fold compared to vehicle.

Our results suggest that the observed increase in mammary cancer progression by daidzein could be through its metabolite equol. To determine whether the increased synthesis of c-Myc, Bcl-2, and Cyclin-D were due to upregulation of gene expression, we performed quantitative RT-PCR analysis following equol treatment using specific primers. There was no significant change in mRNA levels of Bcl-2 or Cyclin D in response to equol. Studies have shown that the key transcription factor c-Myc upregulates eIF4E and eIF4G expression, and in turn becomes elevated by increased translation of c-Myc mRNA. c-Myc has an IRES and is thus, sensitive to elevated eIF4G and eIF4E levels (21). Therefore, the expression of c-Myc in response to equol was determined at both gene and protein levels by qRT-PCR (Fig. 8) and western blotting (Fig. 7). Equol, at low concentrations, upregulated c-Myc gene expression significantly by ~1.5-2-fold (Fig. 8) Increased c-Myc and eIF4F activities are expected to increase protein synthesis, cell cycle progression, and thus, cell proliferation. Therefore, as expected, 25 μM equol induced a ~1.5-fold increase in metastatic cancer cell growth (Fig.9).

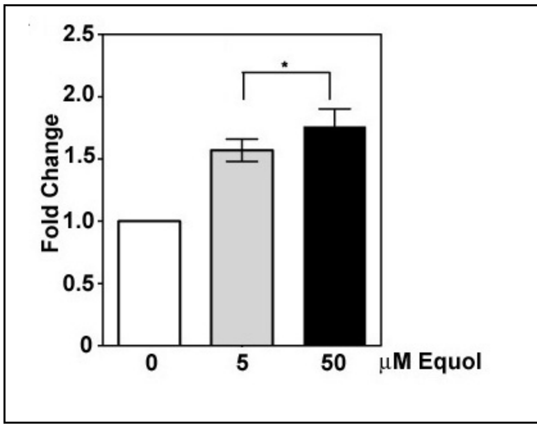

Figure 8. Effect of equol on gene expression of c-Myc. Quiescent MDA-MB-435 cells were treated for 24 h with 5-50 μM equol, and c-Myc expression was quantified by qRT-PCR. Fold changes in *MYC* gene expression from cells treated with equol compared to vehicle. N=3 ± S.D. An asterisk indicates statistical significance of *p*<0.05. p≤0.05.

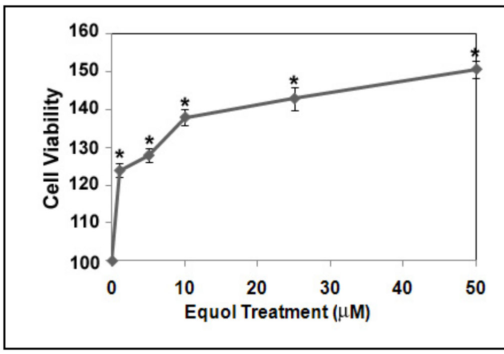

Figure 9. Effect of equol on cell viability. Quiescent metastatic cancer cells were treated with 1-50 μM equol or vehicle for 24 h. Cells were lysed and subjected to a MTT assay. Results are shown relative to vehicle (100%). N=3. Asterisk indicates statistical significance of *P*≤0.05.

Effect of equol on IRES-dependent protein synthesis:

The equol-mediated elevated eIF4G may enhance protein synthesis of pro-cancer molecules by two alternative mechanisms of translation initiation. One potential mechanism is that elevated eIF4G levels may drive cap-dependent eIF4E-associated protein synthesis initiation. The other, is that while eIF4E and the inhibitory protein 4E-BP remain inactive at the cap, the increased eIF4G will upregulate cap-independent protein synthesis initiation that utilizes the 5' leader to recruit the translation machinery through IRESs present in certain mRNAs (14;22). To address the possibility that equol-stimulated eIF4G expression may contribute to cap-independent translation, synthetic *m^7GTP* co-capture assays were performed to isolate cap-bound eIF4E, eIF4G, and 4E-BP from MDA-MB-435 cells treated with equol. Figure 10 demonstrates that total or m^7GTP bound or free eIF4E or 4E-BP levels remained unchanged following equol treatment. This is consistent with our results that showed no effect of equol on phospho- or total eIF4E or 4E-BP protein expression (Fig. 5 A, B), indicating that equol does not affect eIF4E expression or activity. Interestingly, equol treatment significantly decreased the amount of eIF4G co-captured with eIF4E in the m^7GTP beads by ~75% compared to vehicle controls. However, there was a 3-fold increase in eIF4G levels recovered in the total cell lysate and the free pool of eIF4G in the m^7GTP pulldowns (supernatants), indicating that the elevated eIF4G in response to equol is recovered in the cytosol. This result indicates that the Equol-mediated elevated eIF4G is not associated with cap-dependent protein synthesis.

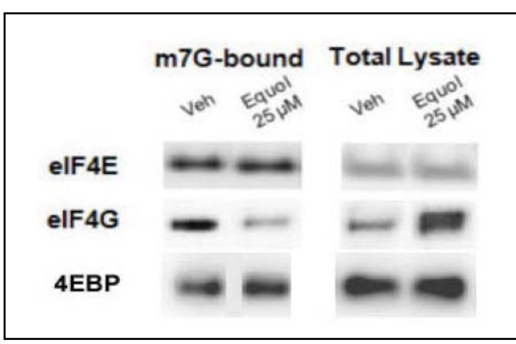

Figure 10. Quiescent metastatic cancer cells were treated with vehicle (Veh) or 25 μM equol for 24 h, lysed, and incubated with Sepharose 4B-conjugated 7-methyl-GTP. The pulldowns were washed and analyzed for eIF4G, eIF4E or 4E-BP associated with total cell lysates, the cap, (m^7GTP), or supernatant (Sup). Representative western is shown (N=3).

In order to determine whether equol was regulated through the excess of eIF4G in the cytosol by cap-independent protein synthesis, we performed dual luciferase assays for cap-dependent and IRES-dependent protein synthesis. This construct was kindly provided by Drs. Deborah Silvera and Robert Schneider (New York University Langone Medical Center, NY) (17). MDA-MB-435 cells were transected with bicistronic reporters containing a cap‐dependent *renilla* luciferase followed by a firefly luciferase driven by *p120 catenin* 5´UTR that contain a IRES. As shown in Fig. 11, equol treatment of MDA-MB-435 cells, specifically increased IRES-driven firefly luciferase activity by 1.6-fold compared to vehicle (P<0.01). These results suggest that the isoflavone daidzein may promote cancer through the metabolite equol. A possible mechanism could be that equol-mediated eIF4G upregulation contributes to non-canonical, eIF4E-independent and thus, 5' 7-methyl-guanosine (M7G) cap-independent, protein synthesis via IRESs.

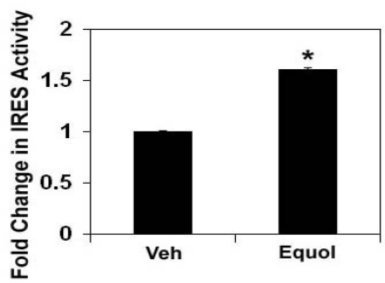

Figure 11. Relative IRES-dependent protein synthesis following equol treatment. MDA-MB-435 cells expressing a plasmid with a cap-dependent *Renilla* luciferase (RLuc) followed by a 5'UTR containing the p120 catenin IRES driving a firefly luciferase (FLuc) or control plasmid without an IRES, were treated with vehicle or equol for 24 h. Cells were lysed, and the relative IRES activity analyzed as 570 nm FLuc/480 nm RLuc. IRES activity was quantified relative to control activity for vehicle or equol treated cells.

Conclusions from Aim 2 and planned experiments: Dietary daidzein may promote breast cancer progression via the metabolite equol. Equol may specifically direct the synthesis of IRES-containing proteins that induce cell survival and cell proliferation and promote cancer malignancy. We plan to perform similar experiments in other breast cancer cell lines during the second year of the award to determine the cell-type dependence of this phenomenon. As proposed, we will also perform sucrose gradient fractionations and polysome analysis of the associated mRNAs following individual or combined soy isoflavones to test the working hypothesis that equol or combined soy isoflavones specifically increase synthesis of mRNAs for pro-cancer molecules with IRESs.

Specific Aim 3: Demonstrate that differential regulation of eIF4E and eIF4G levels by soy isoflavones can directly contribute to breast cancer progression.

This Aim will determine whether soy isoflavones modulate breast cancer progression by direct modulation of eIF4E and (or) eIF4G by investigating the effects on cell cycle progression, cell survival/apoptosis, migration, and invasion in breast cancer cells expressing vector controls or siRNA targeted at eIF4E or eIF4G. These siRNA constructs are available to us from the laboratory of Dr. Robert Schneider (New York University), and this Aim will be completed during the second and third years of the award.

KEY RESEARCH ACCOMPLISHMENTS:

1. Treatment of MDA-MB-231 and SKBR3 cells with individual isoflavones genistein, daidzein and glycitein or in combination (gen:daid:gly) at physiological concentrations did not change eIF4E, eIF4G, or 4E-BP expression.
2. Treatment of MDA-MB-435 cells with combined soy isoflavones increased eIF4E protein expression.
3. MDA-MB-231 or MDA-MB-435 cells did not change eIF4E or eIF4G expression following daidzein treatment, at physiologically relevant concentrations (0-50 µM).
4. The soy isoflavone equol, a metabolite of daidzein with higher bioavailability and greater affinity for ER, significantly increased eIF4G expression in MDA-MB-231 and MDA-MB-435 cells.
5. Concomitant with elevated eIF4G, Equol significantly increased expression IRES containing molecules that are sensitive to eIF4G levels: survivin, Bcl-2, Bcl-XL, c-myc and Cyclin D, and increased cell proliferation.
6. 5' methyl guanosine cap pulldowns to isolate protein synthesis initiation factors demonstrated similar levels of eIF4E and 4EBP at the cap for both vehicle- and equol-treated cells. Interestingly, the elevated eIF4G in response to equol was not associated with the methyl guanosine cap.
7. The elevated cap-independent eIF4G by equol was correlated with increased IRES-dependent protein synthesis by dual luciferase assays.

8. A manuscript was submitted to the peer-reviewed journal, “Journal of Nutritional Biochemistry” on the role of the daidzein metabolite equol on promoting cancer malignancy via specific regulation of protein synthesis of IRES-containing pro-cancer molecules.

REPORTABLE OUTCOMES:

The PI has completed the following milestones in her training program and is on track with the timeline outlined in the original proposal.

Progress towards Ph.D. Degree

1. Completed all of the coursework requirements of the Department of Biochemistry, University of Puerto Rico Medical Sciences Campus.
2. Oct 2011: Successfully completed comprehensive examination for Ph.D. candidacy. She will complete writing her Dissertation proposal and formally present the proposal to her committee on May 03, 2012.

Training

1. June 13-July 01 2011: The PI spent three weeks during summer 2011 at the laboratory of Dr. Leslie A. Krushel at The University of Texas MD Anderson Cancer Center, Houston TX. She learned biochemical techniques to identify cap independent protein synthesis from mRNAs with IRES elements.

Presentation of research at national and international conferences

1. The PI attended and presented at the annual conference of the American Association of Cancer Research (AACR) conference in Orlando, FL, April 2011, and will attend and present a poster at this year’s annual AACR conference in Chicago, IL, April 2012.
2. The PI presented a poster at the annual research conference of the University of Puerto Rico in May 2011, and plans to present another poster at this year’s conference, March 2012.

Abstracts

- American Association of Cancer Research *AACR* 102nd Annual Meeting *2011*. Orlando, FL

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***Manuscript* s**

- **Submitted to the Journal of Nutritional Biochemistry (Feb 21, 2012) "The soy isoflavone equol may increase cancer malignancy via upregulation of eukaryotic protein synthesis initiation factor eIF4G"**
  Columba de la Parra, Elisa Otero-Franqui, Michelle Martinez-Montemayor, and Suranganie Dharmawardhane

  See Appendix

CONCLUSION:

The results demonstrated that individual or combined soy isoflavones genistein, daidzein, and glycitein at physiological concentrations did not affect total or phospho levels eIF4E or the mTOR target 4E-BP that regulates eIF4E activity. Therefore, we conclude that soy isoflavones do not regulate mTOR signaling in breast cancer cells. However, similar to the *in vivo* result from mammary fat pad tumors in nude mice, combined soy isoflavones genistein, daidzein, and glycitein increased eIF4F expression in MDA-MB-435 cells.

The daidzein metabolite equol significantly upregulated eIF4G and c-Myc expression, as well as synthesis of pro-cell survival and proliferation molecules with IRESs. Equol-mediated upregulation of the key cancer promoting transcription factor c-Myc, may contribute to breast cancer progression via multiple mechanisms, that include increased eIF4G expression. Therefore, we conclude that eIF4G upregulation can contribute to non-canonical, eIF4E-independent and thus, 5' 7-methyl-guanosine (M7G) cap-independent, protein synthesis of IRES containing mRNAs. Equol may specifically direct the synthesis of IRES-containing proteins that induce cell survival and cell proliferation and promote cancer malignancy. Future experiments will demonstrate that differential regulation of eIF4G levels by soy isoflavones directly contributes to breast cancer progression through selective knockdown of eIF4G.

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APPENDICES

Control/Tracking Number: 11-A-3970-AACR
Activity: Abstract Submission
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Equol, a metabolite of the soy isoflavone daidzein, may promote cancer metastasis via regulation of eukaryotic protein synthesis initiation factors

Short Title:
Soy regulates protein synthesis

Author Block: Columba De La Parra Simenthal[1], Elisa Otero-Franqui[1], Michelle Martinez-Montemayor[2], Surangani F. Dharmawardhane Flanagan[1]. [1]Univ. of Puerto Rico Med. Science Campus, San Juan, PR; [2]Universidad Central del Caribe, Bayamon, PR

Abstract:
The cancer preventive role of the major soy isoflavones genistein and daidzein is well established. The purpose of our investigation is to understand the role of dietary soy isoflavones in cancer metastasis. We recently reported that dietary daidzein increased mammary tumor growth and metastasis in a nude mouse model with tumors established from MDA-MB-435 metastatic cancer cells. Molecular analysis of the primary tumors demonstrated elevated expression of eukaryotic protein synthesis initiation factors eIF4G and eIF4E (Clin Exp Metastasis. 2010, 27:465-480). These eIF4F family initiation factors are overexpressed in advanced cancers and have been implicated in translational control of cancer by specific synthesis of cancer promoting proteins. Western blotting of the primary tumors following daidzein or combined soy isoflavones demonstrated increased expression of mRNAs with long structured 5'UTRs that are sensitive to elevated eIF4F initiation factors, such as p120 catenin, survivin, Bcl-2, Bcl-XL, and VEGF. Gut microflora of mice and humans metabolizes daidzein to form equol that has higher bioavailability and greater affinity for estrogen receptors (ER) than daidzein. In vitro studies using MDA-MB-435 (ER-) and MDA-MB-231 (ER β+) metastatic cancer cell lines demonstrated that the effect on protein synthesis by daidzein was exerted via its metabolite equol. Western blotting of cell lysates treated with equol (1-50 microM) for 24 hours demonstrated that equol significantly increased total and phospho-eIF4G in a concentration-dependent manner. The protein levels of eIF4E and its inhibitory 4E-bidning protein (4E-BP) remained unchanged while equol decreased phospho-4EBP. In advanced cancers, increased levels of hypophosphorylated 4E-BPs, in conjunction with elevated levels of eIF4G, is thought to function as a switch to promote cap-independent translation. Enhanced eIF4G levels can specifically initiate translation of mRNAs that contain an internal ribosome entry site (IRES) to which eIF4G may bind directly. Accordingly, in MDA-MB-435 cells, equol significantly increased expression of c-myc, an mRNA with an IRES site. These results indicate that dietary soy may promote cancer through equol by specifically initiating cap-independent synthesis of proteins relevant for cancer malignancy.
:

Author Disclosure Information: **C. De La Parra Simenthal:** None. **E. Otero-Franqui:** None. **M. Martinez-Montemayor:** None. **S.F. Dharmawardhane Flanagan:** None.
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AACR ANNUAL MEETING March 31- April 4, 2012 Chicago, IL ABSTRACT SUBMITTER

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Increased expression of eukaryotic protein synthesis initiation factor eIF4G by the daidzein metabolite equol may contribute to breast cancer malignancy

Short Title:
Equol in cancer malignancy

Author Block: Columba de la Parra, Elisa Otero-Franqui, Suranganie Dharmawardhane. University of Puerto Rico, Medical Sciences Campus, San Juan, PR

Abstract:
The role of dietary soy in cancer has been the subject of intense investigation and is thought to be cancer preventive. However, the beneficial effects of soy on established breast cancer is controversial. We recently demonstrated that dietary daidzein and combined soy isoflavones (genistein, daidzein, and glycitein) promoted breast cancer progression in a nude mouse model by increasing both primary mammary tumor growth and metastasis. Dietary daidzein significantly upregulated cancer promoting molecules including eukaryotic protein synthesis initiation factors (eIF) eIF4G and eIF4E. Herein, using tumors from mice treated orally with daidzein, we show that increased eIF expression is associated with expression of mRNAs with long structured 5' untranslated regions and internal ribosomal entry sites (IRES) that are sensitive to eIF4E and eIF4G levels. We then tested the hypothesis that daidzein upregulates protein synthesis initiation in breast cancer, but found that daidzein treatment did not affect the levels of eIFs in the same MDA-MB-435 cell line *in vitro*. Therefore, we tested the effect of equol that is metabolized from daidzein by the gut bacteria in mice and humans. Results show that equol specifically upregulated eIF4G, but not eIF4E, in MDA-MB-435 and MDA-MB-231 human breast cancer cells. Equol treatment also increased gene and protein expression of c-MYC and protein expression of other cell survival and proliferation promoting molecules with IRES sites. The elevated eIF4G in response to equol was not associated with eIF4E in a cap binding co-capture assay. Therefore, upregulation of eIF4G by equol may regulate cap-independent protein synthesis initiation resulting in cancer cell survival, proliferation, and thus, tumor progression.
This research was supported by grant numbers US Army/BCRP W81XWH-11-1-0199 to CD, to NIH/NIGMS SC3GM084824 to SD
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Title: The soy isoflavone equol may increase cancer malignancy via upregulation of eukaryotic protein synthesis initiation factor eIF4G

Article Type: Research Article

Keywords: breast cancer; equol; eIF4G; cap-independent protein synthesis; IRES-dependent protein synthesis

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Order of Authors: Columba de la Parra, B.S., M.S.; Elisa Otero-Franqui, M.D.; Michelle Martinez-Montemayor, Ph.D.; Suranganie Dharmawardhane, Ph.D.

Abstract: The role of dietary soy in cancer has been the subject of intense investigation and is thought to be cancer preventive. However, the beneficial effects of soy on established breast cancer is controversial. We recently demonstrated that dietary daidzein or combined soy isoflavones (genistein, daidzein, and glycitein) increased primary mammary tumor growth and metastasis in a nude mouse model. Cancer promoting molecules, including eukaryotic protein synthesis initiation factors (eIF) eIF4G and eIF4E, were upregulated in mammary tumors from mice that received dietary daidzein. Herein, we show that increased eIF expression in these tumor extracts is associated with expression of mRNAs with internal ribosome entry sites (IRESs) that are sensitive to eIF4E and eIF4G levels. Results with MDA-MB-231 and MDA-MB-435 metastatic cancer cell lines show that the effect of daidzein on eIFs in vivo can be recapitulated by the daidzein metabolite equol. In vitro, equol, but not daidzein, upregulated eIF4G without affecting eIF4E or its regulator 4E binding protein (4E-BP) levels. Similar to increased tumor growth by dietary daidzein, equol increased metastatic cancer cell proliferation. Equol also increased c-Myc levels, as well as expression of IRES containing cell survival and proliferation promoting molecules. The elevated eIF4G in response to equol was not associated with eIF4E or 4E-BP at the 5' cap, in a co-capture assay. In dual luciferase expression assays, IRES-dependent protein synthesis was increased by equol treatment. Therefore, upregulation of eIF4G by equol may result in increased translation of pro-cancer mRNAs with IRESs, and thus, promote cancer malignancy.

The soy isoflavone equol may increase cancer malignancy via upregulation of eukaryotic protein synthesis initiation factor eIF4G

Columba de la Parra[1], Elisa Otero-Franqui[1], Michelle Martinez-Montemayor[2], Suranganie Dharmawardhane[1]*

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Running Title: Equol increases eIF4G expression in cancer cells

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Key Words: breast cancer; equol; eIF4G; cap-independent protein synthesis; IRES-dependent protein synthesis

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Abstract

The role of dietary soy in cancer has been the subject of intense investigation and is thought to be cancer preventive. However, the beneficial effects of soy on established breast cancer is controversial. We recently demonstrated that dietary daidzein or combined soy isoflavones (genistein, daidzein, and glycitein) increased primary mammary tumor growth and metastasis in a nude mouse model. Cancer promoting molecules, including eukaryotic protein synthesis initiation factors (eIF) eIF4G and eIF4E, were upregulated in mammary tumors from mice that received dietary daidzein. Herein, we show that increased eIF expression in these tumor extracts is associated with expression of mRNAs with internal ribosome entry sites (IRESs) that are sensitive to eIF4E and eIF4G levels. Results with MDA-MB-231 and MDA-MB-435 metastatic cancer cell lines show that the effect of daidzein on eIFs *in vivo* can be recapitulated by the daidzein metabolite equol. *In vitro,* equol, but not daidzein, upregulated eIF4G without affecting eIF4E or its regulator 4E binding protein (4E-BP) levels. Similar to increased tumor growth by dietary daidzein, equol increased metastatic cancer cell proliferation. Equol also increased c-Myc levels, as well as expression of IRES containing cell survival and proliferation promoting molecules. The elevated eIF4G in response to equol was not associated with eIF4E or 4E-BP at the 5' cap, in a co-capture assay. In dual luciferase expression assays, IRES-dependent protein synthesis was increased by equol treatment. Therefore, upregulation of eIF4G by equol may result in increased translation of pro-cancer mRNAs with IRESs, and thus, promote cancer malignancy.

1. Introduction

Isoflavones found primarily in legumes, and particularly in soy, are a major class of phytoestrogens that are structurally and/or functionally similar to 17β estradiol [1]. These compounds have received increasing attention for their potential estrogenic or antiestrogenic effects, leading to concerns surrounding the use of phytoestrogen supplements in breast cancer patients who may overexpress estrogen receptors in the tumor tissue [2]. Since soy foods have anticancer effects at early stages of carcinogenesis, most studies have focused their investigation on prevention of breast cancer risk reduction by soy isoflavones [3]. Although many studies have focused on the effects of soy consumption in breast cancer, the benefits of soy foods as chemopreventives for established breast cancer or as substitutes for hormone replacement therapies remain controversial [1,3-5].

The second most prominent isoflavone found in soybeans and soy products is the aglycone form daidzein. Intestinal bacteria are central to the absorption and metabolism of isoflavones. After oral ingestion, glucosidases metabolize the β-glycosidic isoflavone daidzin into their corresponding bioavailable aglycone daidzein (6). Daidzein can be further metabolized to equol; before final absorption, the intestinal microflora converts daidzein to equol or O-desmethylangolensin (O-DMA) (Fig. 1). Rodents are efficient producers of equol. However, not all humans have the gut microflora necessary to convert daidzein to equol, and approximately 30–50% of humans are equol producers. The proportion of equol producers also vary with demographic, lifestyle factors, and ethnicity; and certain populations (e.g. Chinese) have been shown to be high equol producers [5,7]. This variation in equol production

may explain the discrepancies found in epidemiological studies on the risks or benefits of dietary soy [5,6,8-10].

Unlike the metabolite O-DMA, which has low biological activity [6,11], equol is structurally similar to estrogen with eighty times more estrogen receptor (ER)-β affinity than its precursor daidzein [11-13]. Equol has been implicated with decreased prostate cancer cell proliferation and prostate cancer risk by acting as an antagonist for dihydrotesterone [14,15]. In ER (+) T47D and MCF-7 human breast cancer cells, equol increased estrogenic activity and cell proliferation but dietary equol did not affect tumor growth in nude mice [16-19]. Dietary daidzein also failed to reduce chemically-induced mammary tumor growth in rats that demonstrated ~1 μM equol in the serum [20]. Others have shown that equol inhibited growth and invasion of ERα (-) ERβ (+) human breast cancer cells and induced cell cycle arrest and apoptosis [14,21-23]. However, caution must be exercised when interpreting *in vitro* studies because the inhibitory effects of equol in breast cancer cells were observed at concentrations ranging from 50-100 μM [14,22,23], while low physiologically relevant concentrations of equol ($\leq 1\mu$M) increased breast cancer cell proliferation [17,24]. Moreover, dietary soy, where genistein, daidzein, and equol were detected in serum samples, increased mammary epithelial cell proliferation of human subjects [25]. Therefore, the association between equol production and cancer risk in humans remain to be adequately characterized [8,26,27]. Overall, benefits from soy intake are associated with ER (+) breast cancer and the effect of equol or soy isoflavones on ER (-) breast cancers or established aggressive breast cancers remains to be clarified [5,28,29].

Our recent data, using ER (-) highly metastatic MDA-MB-435 human cancer cells, reported that dietary daidzein and soy isoflavones (daidzein:genistein:glycitein, 5:4:1) increased mammary tumor growth and metastasis in nude mice [30]. PCR analysis of mammary tumors demonstrated that dietary daidzein upregulated the expression of a number of genes that regulate cell proliferation and survival including *CCND1, CTNNB1, GRB2, JUN, MAPK1,* and *IRS1*. Of note was the significant upregulation of eukaryotic initiation factor 4G (*EIF4G1)* and increased eIF4G and eIF4E protein levels in tumors following daidzein diets [30]. Increased levels of eIF4F family members such as eIF4E, G, and B have been implicated with specific translation of tumor survival and malignancy-promoting proteins that have mRNAs with long structured 5' untranslated regions (UTR) and (or) internal ribosome entry sites (IRES) [31-33].

The present study was initiated to test the hypothesis that dietary daidzein promotes cancer progression via specific synthesis of cancer promoting molecules that are sensitive to elevated eIF4G levels. We show that the isoflavone daidzein may promote cancer through the metabolite equol. Equol-mediated eIF4G upregulation can contribute to non-canonical, eIF4E-independent and thus, 5' 7-methyl-guanosine (M^7G) cap-independent, protein synthesis via IRES sites [33,34]. Therefore, equol may specifically direct the synthesis of IRES-containing proteins that induce cell survival and cell proliferation and promote cancer malignancy.

2. Methods and Materials

2.1. Cell culture

Metastatic variant of MDA-MB-435 (ER−) (gift of Dr. Danny Welch, The University of Kansas Cancer Center) and MDA-MB-231 (ER*α*−, ER*β*+) metastatic human breast cancer cells (American Type Culture Collection, Manassas, VA, USA) were maintained in complete culture medium: Dubelco's Modified Eagle Medium (DMEM) (Invitrogen, Houston, TX) supplemented with 10% fetal bovine serum (FBS; DMEM; Invitrogen, Houston, TX) at 37 °C in 5% CO_2.

2.2. Cell treatment

Quiescent metastatic cancer cells were treated with 0 (vehicle, 0.1% DMSO), 1, 5, 10, 25, or 50 µM of isoflavone daidzein (LC Laboratories, Woburn,MA) or metabolite (R,S) Equol (LC Laboratories, Woburn, MA) in DMEM and 5% FBS media for 24 or 48 h.

2.3. Tumor model

The tumors were derived from our previous study [30]. Briefly, female athymic nu/nu mice, 5 week old (Charles River Laboratories, Wilmington, MA) were inoculated at the mammary fat pad with green fluorescent protein (GFP) tagged-MDA-MB-435 cells. After 1 week of tumor inoculation, vehicle (10% ethanol, 90% corn oil), 10 mg/kg body weight (BW) of daidzein, or combined soy isoflavones 10 mg/kg BW genistein, 9 mg/kg BW daidzein, and 1 mg/kg BW glycitein were administered 3 times a week by oral gavage for 11 weeks. Following necropsy, mammary tumors were excised and stored snap frozen in liquid nitrogen.

2.4. Western blotting

Cells and tumors were lysed and western blotted, as described in [30]. Primary antibodies to eIF4E, phospho (P)-eIF4E ^{Ser209}, eIF4G , p-eIF4G ^{Ser1108}, 4E-BP1, p4E-BP1 $^{Thr37/46}$, c-Myc , p120 catenin, p-p120$^{Thr\ 916}$ catenin, survivin, Bcl-XL, Bcl2, vascular endothelial growth factor (VEGF), Cyclin D, and β actin (Epitomics, Burlingame, CA, Cell Signaling, Danvers, MA, Sigma-Aldrich Comp., St Louis, MO) were used. Data from mouse mammary tumors were normalized to GFP expression to ensure quantification of proteins from GFP-MDA-MB-435 cells using anti-GFP antibody (Abcam, Cambridge, MA). The integrated density of positive bands was quantified using Image J software, as described in [30].

2.5. Real-Time reverse transcriptase polymerase reaction (RT-PCR) analysis

As described in [35], real-time quantitative RT-PCR analysis was performed from cells treated with vehicle or equol for 24 h**.** Total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Valencia, CA). RNA concentration was detected using a NanoDrop (Thermo Scientific, Wilmington, DE). RNA (0.5 μg) was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR primers were as follows. *MYC*: forward, 5′-TTCTCAGAGGCTTGGCGGGAAA-3′, reverse, 5′ TGCCTCTCGCTGGAATTACTACA-3′. B2M (control): forward, 5′-GGCTATCCAGCGTACTCCAAA-3′, reverse, 5′- CGGCAGGCATACTCATCTTTTT-3′. Real-time reactions were performed using iQ SYBR-Green PCR Master Mix (Bio-Rad, Hercules, CA). The amplification reaction was performed for 40 cycles (10s at 95 °C,

30s at 59°C, and 30s at 72 °C). The fold change was determined by the 2 $^{\Delta\Delta CT}$ method as described in [30,35].

2.6. Cell viability assay

Cell viability was determined by the CellTiter 96® Aqueous One Solution Cell Proliferation Assay containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and phenazine methosulfate (PMS) as active reagents, according to manufacturer's instructions (Promega, Madison, WI), and as described in [35]. Briefly, quiescent 1 x 10^5 MDA-MB-435 cells were added to the wells of a 96-well plate and treated for 24 h with vehicle, 1, 5, 10, 25, or 50 μM equol. Following equilibration, 20 μl/well of combined MTS/PMS solution was added and the absorbance of the formazan product measured at 570 nm using an ELISA plate reader.

2.7. Cap affinity chromatography

Cell lysates, following vehicle or 25 μM equol treatment for 24 h, were incubated with 7-Methyl-GTP (m7GTP) Sepharose 4B or control beads (Amersham Biosciences) for 1 h at 4^0C, as described in [36]. Total lysates, washed beads following m^7GTP co-capture, and the supernatants were western blotted for eIF4E, 4E-BP, or eIF4G.

2.8. Luciferase reporter assays

MDA-MB-435 cells were transfected with a bicistronic reporter system (kind gift of Dr. Robert Schneider, New York University Langone Medical Center) or control

plasmid containing the luciferase constructs without an IRES, using Lipofectamine 2000 (Invitrogen), as per manufacturer's directions. As described in [32], this plasmid contains a cap-dependent *Renilla* luciferase (RLuc) followed by a 5'UTR containing the p120 catenin IRES driving a firefly luciferase (FLuc). 24 h following transfection, cells were treated with equol for an additional 24 h. The relative IRES activity was analyzed as 570 nm FLuc/480 nm RLuc in a luminometer using a dual luciferase assay kit (Promega, Corp., Madison, WI), according to manufacturer's instructions.

2.9. Statistical analysis

Data was analyzed and reported as mean ± SEM in triplicate. Statistical analyses were done using Microsoft Excel and GraphPad Prism. Differences between means were determined using Student's *t*-Test and $P \leq 0.05$ considered significant.

3. Results

3.1. Dietary daidzein upregulates expression of eIF4G and eIF4E and increased translation of mRNAs with IRES sites in vivo, but not in vitro

We recently reported that daidzein increased mammary tumor growth and metastasis in nude mice with mammary tumors established from the ER (-) highly metastatic human cancer cell line MDA-MB-435. Mammary tumors from mice treated with daidzein diets demonstrated a significant 2-3-fold upregulation of *EIF4G1* gene and protein expression and a ~7.0-fold increase in eIF4E protein levels, compared to vehicle controls. Combined soy treatment resulted in 1.8 fold increase in *EIF4E* gene and a 2.5-fold increase in protein expression [30]. High levels of eukaryotic initiation factors,

specifically eIF4G1, have been correlated with increased cap independent translation of specific mRNAs that contain IRESs and long structured 5'UTRs [32,33,37]. To investigate the effect of the overexpressed eIF4F complex on translation of mRNAs sensitive to elevated eIF4F initiation factors, we analyzed protein expression levels of pro-survival, -angiogenesis, and –proliferation of molecules known to have mRNAs with long UTRs and/or IRESs [32,33,38], from primary tumors of mice following daidzein or combined soy (genistein:daidzein:glycitein, 5:4:1) diets. As shown in Figure 2, the pro-survival proteins survivin (2-fold), Bcl2 (2.2-fold), and Bcl-XL (2.3-fold), and total and active p120 catenin (4- and 8-fold) were significantly ($P \leq 0.05$) increased in tumors following daidzein diets. Combined soy also demonstrated significant increases in expression of Bcl-XL (4-fold), and VEGF (2.3-fold). However, the expression of β-actin, a constitutively expressed mRNA with a short 5'UTR was not affected by dietary soy isoflavones. Both actin and GFP expression were used as standards for the analysis of fold differences of IRES containing molecules compared to vehicle controls to ensure analysis of GFP-MDA-MB-435 cells.

This data implicate dietary daidzein in eIF4F-controlled translation of proteins that regulate cancer progression. Therefore, the molecular mechanisms of daidzein action were further investigated *in vitro* using the same cell line from the *in vivo* study, ER (-) MDA-MB-435 cells and the ERβ (+) metastatic breast cancer cell line MDA-MB-231, following vehicle or daidzein treatment at concentrations ranging from 0-50 μM. These concentrations are within the range of 1-10 μM that has been shown to accumulate in the circulation following consumption of soy products [39]. However, we did not detect any significant changes in eIF4E, p-eIF4E, eIF4G, or p-eIF4G in both cell

lines following 24 or 48 h treatment of daidzein at all concentrations tested. There were slight increases in eIF4G and p-eIF4G in MDA-MB-231 cells treated with 25 and 50 μM daidzein; nevertheless, these increases were not statistically significant (Fig. 3).

3.2. The daidzein metabolite equol upregulates expression of eIF4G and c-Myc and increased translation of mRNAs with IRESs in vitro

Daidzein can be further metabolized to equol (70%) and O-desmethylangolensin (O-DMA) (5-20%). In rodents, equol is the major circulating metabolite and all rodents are equol producers [6,11]. Therefore, we reasoned that the daidzein effects on MDA-MB-435 metastatic cell lines *in vivo* may be due to the metabolite equol. MDA-MB-435 and MDA-MB-231 cells were treated with (R,S)-Equol at different concentrations (0-50 μM) and tested for eIF4E and eIF4G expression by western blotting. Figure 4 demonstrates that similar to dietary daidzein in mice, equol increased the expression of total and p-eIF4G in a concentration-dependent manner. p-eIF4G levels paralleled the elevated eIF4G levels; therefore, equol may not specifically affect phosphorylation of eIF4G, only eIF4G expression. In MDA-MB-435 cells, equol at 25 μM and 50 μM significantly increased eIF4G expression by ~1.8-fold ($P \leq 0.05$) compared to vehicle controls. The increase in eIF4G in the MDA-MB-231 cell line was more modest (~1.3-fold), but consistent at similar concentrations (>10 μM). It is possible that the presence of ERβ in the MDA-MB-231 cell line may exert a differential effect on equol-mediated eIF4G expression. The protein levels of eIF4E and its inhibitory protein 4E-BP remained unchanged at all concentrations of equol tested, in both cell lines, indicating a specific effect on eIF4G expression and not eIF4E expression or regulation.

Studies have shown that the key transcription factor c-Myc upregulates eIF4E and eIF4G expression, and in turn becomes elevated by increased translation of c-Myc mRNA. c-Myc has an IRES site and is thus, sensitive to elevated eIF4G and eIF4E levels [40]. Therefore, the expression of c-Myc in response to equol was determined at both gene and protein levels by qRT-PCR and western blotting. Equol upregulated c-Myc significantly by ~2-fold at concentrations as low as 1 μM (Fig.5 A,B). Increased c-Myc and eIF4F activities are expected to increase protein synthesis, cell cycle progression, and thus, cell proliferation. Therefore, as expected, 25 μM equol treatment induced a ~1.5-fold increase in metastatic cancer cell growth (Fig.5C).

A recent study from ER (-) inflammatory breast cancer cells demonstrated that overexpression of eIF4G with no changes in the cap binding protein eIF4E and its negative regulator 4E-BP1 can result in increased cap-independent protein synthesis of IRES-containing mRNAs [32]. To determine whether increased eIF4G in response to equol may drive IRES-dependent protein synthesis, we tested the expression of IRES-containing mRNAs that regulate cancer cell survival and proliferation [31-34,40-44]. Figure 6 demonstrates that similar to the effect of dietary daidzein in MDA-MB-435 tumors, equol, at 10 and 25 μM, upregulated protein expression of survivin, Bcl-2, and Bcl-XL in the MDA-MB-435 cells by ~1.3-1.7-fold compared to vehicle. Cyclin D (*CCND1*) which was upregulated by 3.0-fold in MDA-MB-435 mammary tumors following dietary daidzein [30], responded *in vitro* to equol treatment (25 μM) by a similar 2.0-fold increase in protein expression ($P \leq 0.05$) (Fig. 6A,B), but not in gene expression (data not shown).

The equol-mediated elevated eIF4G can enhance two alternative mechanisms of translation initiation. One potential mechanism is that elevated eIF4G levels may drive cap-dependent eIF4E-associated protein synthesis initiation. The other, is that while eIF4E and the inhibitory protein 4E-BP remain inactive at the cap, the increased eIF4G will upregulate cap-independent protein synthesis initiation that utilizes the 5' leader to recruit the translation machinery through IRESs present in certain mRNAs [33,38] (Fig. 7D). To address the possibility that equol-stimulated eIF4G expression may contribute to cap-independent translation, synthetic m^7GTP co-capture assays were performed to isolate cap-bound eIF4E, eIF4G, and 4E-BP from MDA-MB-435 cells treated with equol. Fig. 7A demonstrates that total or m^7GTP bound or free eIF4E or 4E-BP levels remain unchanged following equol treatment. This is consistent with our results that showed no effect of equol on phospho- or total eIF4E or 4E-BP protein expression (Fig. 4), indicating that equol does not affect eIF4E expression or activity. Interestingly, equol treatment significantly decreased the amount of eIF4G co-captured with eIF4E in the m^7GTP beads by ~75% compared to vehicle controls. However, there was a 3-fold increase in eIF4G levels recovered in the total cell lysate and the free pool of eIF4G in the m^7GTP pulldowns (supernatants), indicating that the elevated eIF4G in response to equol is recovered in the cytosol. This result indicates that the Equol-mediated elevated eIF4G is not associated with cap-dependent protein synthesis.

To determine whether the excess eIF4G in the cytosol following m^7G cap pulldowns was regulating cap-independent protein synthesis, we performed dual luciferase assays for cap-dependent and IRES-dependent protein synthesis. As shown in Fig. 7C, equol treatment, of MDA-MB-435 cells, specifically increased IRES-driven

firefly luciferase activity by 1.6-fold compared to vehicle ($P \leq 0.01$). Taken together, our results strongly suggest that equol-mediated elevated eIF4G specifically directs cap-independent protein synthesis initiation of IRES-containing cell survival and pro-proliferation molecules, while eIF4E remains inactive at the 5' cap, bound to 4E-BP (Fig. 7D).

4. Discussion

Previously, we reported that dietary daidzein and combined soy isoflavones increase mammary tumor growth and metastasis [30]. Since daidzein treatment to mice with MDA-MB-435 xenografts upregulated eIF4G in mammary tumors, we investigated a role for dietary daidzein in cancer progression via upregulated protein synthesis initiation. Results presented herein, demonstrate that the effect of daidzein on eIF4G expression could not be recapitulated *in vitro* with daidzein, but only with the major daidzein metabolite in rodents, equol. Since, eIF4G is elevated in a number of human cancers and shown to contribute to their malignancy [32,33], the upregulated eIF4G in response to equol may be a major effector of the increased cancer progression observed *in vivo,* by dietary daidzein [30]. Although dietary daidzein significantly increased eIF4E protein levels and combined soy increased both gene and protein expression of eIF4E, equol treatment *in vitro* did not affect eIF4E or its negative regulator 4E-BP expression or activity. Therefore, soy consumption may have more profound effects on protein synthesis initiation in cancer cells than the effects of equol described in this report.

Equol has been implicated with daidzein activities *in vivo* following ingestion of

soy foods, and has been shown to be more potent than daidzein *in vitro* (8;45;46). Equol is a chiral molecule, capable of existing in two enantiomeric forms: R-(+) equol and S-(-) equol; the latter is the natural diasteromer produced by intestinal bacteria [6,11]. Both equol enantiomers show better uptake and have higher bioavailability (65-83%) than the isoflavones dadizein (30-40%) or genistein (7-15%) (47). Both enantiomers bind ERs, with R-equol showing a preference for ERβ [13]. The racemic (R,S)-equol used for this study demonstrates a more pronounced response to equol in upregulation of eIF4G in ER (-) MDA-MB-435 cells compared to ERβ (+) MDA-MB-231 cells. This result suggests that the effect of equol on eIF4G expression is not ER dependent, or that the presence of ERβ has a protective effect on the cancer promoting action of equol. To our knowledge, *EIF4G* does not have an estrogen response element (ERE) and therefore, cannot be directly under the regulation of ER.

Equol treatment at low dietary concentrations, demonstrated a dramatic effect on c-Myc expression. *MYC* has an ERE and its expression is known to be regulated by estrogen and estrogen mimetics [48], as well as by a plethora of signaling pathways and mechanisms [49]. The c-Myc transcription factor is one of the most important somatically mutated oncogenes in human cancer and confers a selective advantage to cancer cells by promoting protein synthesis, proliferation, cell survival, differentiation, genetic instability, angiogenesis, hypoxia-mediated cancer progression, and metastasis [33,44,49-54]. Herein, we show that equol treatment of MDA-MB-435 cells recapitulated the previously reported increase in MDA-MB-435 tumor growth in response to dietary daidzein [30]. This result strongly suggests that elevated cancer promoting molecules in response to equol can increase cell survival and proliferation contributing to the

observed effects of enhanced tumor growth and metastasis by dietary daidzein and soy isoflavones.

In addition to a large number of pro-cancer molecules, equol-mediated c-Myc upregulation can result in increased transcription of eIF4G and eIF4E [55,56]. c-Myc also has an IRES site and is therefore, sensitive to elevated eIF4G and eIF4E levels [40]. eIF4F family initiation factors that include eIF4E and eIF4G are overexpressed in advanced cancers and have been shown to be essential for translation of a subset of proteins that regulate cellular bioenergetics, survival, and proliferation [32,33,57,58]. During canonical initiation of translation, eIF4E binds the 5′ m^7G cap of mRNAs and delivers them to the eIF4F translation initiation complex. eIF4G plays a central role in the assembly of this pre-initiation complex by acting as a scaffolding protein that recruits the 3' poly A tail of mRNA, and interacts with other initiation factors including eIF4E. The eIF4F complex assembly is rate limiting for initiation and is dependent upon eIF4E availability [34]. eIF4G can also initiate eIF4E- and cap-independent protein synthesis by binding to IRES sites in certain mRNAs and recruiting ribosomes and other eIF4F complex members [32,33,59].

Thus, the initial equol-mediated elevation of *MYC* and *EIF4G1* by regulation of gene expression is predicted to result in further synthesis of eIF4G and c-Myc via IRES-driven mechanisms, where eIF4G itself has an IRES site [38,60]. IRES containing cyclin D1 and c-Myc upregulation are hallmarks of cancer that have been directly associated with eIF4G upregulation [32,33,38,61-63]. The cell survival genes survivin, Bcl-2, and Bcl-XL are sensitive to eIF4G levels, have IRES sites, and are elevated in aggressive cancers [64-67]. p-120 catenin and phosphorylated p^{T916}-p120 catenin have been

shown to stabilize the E-cadherin axis at cell adhesions and implicated in regulation of Rho GTPase function leading to increased cancer cell invasion [68]. The E-cadherin axis is lost in metastatic cancer cells that have undergone epithelial to mesenchymal transition (EMT) such as the MDA-MB-231 and MDA-MB-435 cells used in our study. Therefore, the elevated p120 catenin in response to equol may contribute to cancer progression via enhanced nuclear transcription regulated by free p120 catenin [69].

Our results that demonstrate elevated eIF4G in response to equol strongly suggest that this upregulation may increase synthesis of IRES containing mRNAs. First, we show that a majority of the upregulated eIF4G is phosphorylated, thus, suggesting it is functional and available for kinase activity in the cytosol. The functional consequence of phosphorylation of eIF4G in translation is not well established. Recent reports have implicated phosphorylation of eIF4G by PAK2 in inhibition of cap-dependent translation but not IRES-driven translation [70], thus implicating elevated p-eIF4G in response to equol in IRES-dependent protein synthesis initiation. Second, our results from mouse mammary tumors following daidzein diets, and breast cancer cells following equol treatment, corroborate the hypothesis that elevated eIF4G by dietary daidzein, and presumably via the metabolite equol, increases the translation of specific mRNAs with IRESs. Third, we show that the equol-regulated eIF4G is not associated with eIF4E at the 5’ cap but is recovered as a free cytosolic pool. Fourth, dual luciferase assays demosntrated that equol treatment preferentially increases the expression of an IRES-driven firefly luciferase relative to a cap-dependent Renilla luciferase.

In conclusion, we have shown that the daidzein metabolite equol may be a potent regulator of the cancer promoting effects of dietary daidzein. Therefore, consumption of

soy foods may not be advisable for patients with ER (-) breast cancer; however, more research needs to be conducted prior to definitive dietary recommendations.

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Figure Captions

Fig. 1. Daidzein is metabolized to equol by intestinal bacteria.

Fig. 2. Effect of soy isoflavones on protein expression in mammary fat pad tumors from mice treated with vehicle, genistein, daidzein, or soy isoflavones (genitein:daidzein:glycitein (5:4:1). Mammary fat pad tumors established from GFP-MDA-MB-435 cells, from the study described in (30), were lysed and the proteins extracted. (A) Representative western blots from tumor extracts immunostained for eIF4E and eIF4G and cancer promoting proteins with IRES sites known to be sensitive to eIF4F levels. (B) Fold changes of protein expression compared to vehicle as calculated from the integrated density of positive bands from western blots and normalized with actin and GFP expression. Values show mean ± SEM (N=3). An asterisk indicates statistical significance of $P \leq 0.05$.

Fig. 3. Effect of daidzein on total and phospho (p) eIF4E and eIF4G expression in MDA-MB 435 and MDA-MB 231 cells. Quiescent cells were treated with vehicle or daidzein (0-50 μM) in 5% serum for 24 h, lysed, and western blotted with mono-specific antibodies. Left, representative western blots of MDA-MB 435 cell lysates. (Right, representative western blots of MDA-MB 231 cell lysates. Blots are representative of 3 biological replicates.

Fig. 4. Effect of equol on total and phospho (p) eIF4E, eIF4G, and 4E-BP expression in MDA-MB 435 and MDA-MB 231 cells. Quiescent cells were treated with vehicle or

equol (0-50 μM) for 24 h, lysed, and western blotted with mono-specific antibodies. (A-B) Representative western blots and fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of MDA-MB 435 cell extracts. (C-D) Representative western blots and fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of MDA-MB 231 cell extracts. Values show mean ± SEM (N=3). An asterisk indicates statistical significance of $p<0.05$.

Fig. 5. Effect of equol on gene and protein expression of c-Myc. Quiescent MDA-MB-435 cells were treated for 24 h with 5-50 μM equol, and c-Myc expression was quantified by qRT-PCR and western blotting. (A) Fold changes in *MYC* gene expression from cells treated with equol compared to vehicle. (B) Top, representative western blot for cell lysates following equol or vehicle treatment. Bottom, fold changes in c-Myc protein expression compared to vehicle as calculated from the integrated density of positive bands from western blots, and normalized with actin expression. (C) Effect of equol on cell viability. Quiescent MDA-MB-435 cells were treated with 1-50 μM equol or vehicle for 24 h. Cells were lysed and subjected to a MTT assay. Results are shown relative to vehicle (100%). N=3 for all experiments. A single asterisk indicates statistical significance of $P\leq 0.05$.

Fig. 6. Expression of cell survival proteins following equol treatment. Quiescent MDA-MB-435 cells were treated with vehicle or 1-50 μM equol for 24 h, lysed, and western blotted with specific antibodies to survivin, Bcl-XL, Bcl2, and Cyclin D. (A)

Representative western blots. (B) Fold changes relative to actin were calculated by Image J analysis of positive bands from equol treatments (1-25 μM) compared to vehicle controls. N=3. An asterisk indicates statistical significance of $p<0.05$.

Fig. 7. Potential regulation of IRES-dependent protein synthesis initiation by equol. (A) 7-Methyl-GTP (m7G) co-capture assays. Quiescent MDA-MB-435 cells were treated with vehicle or 25 μM equol for 24 h, lysed, and incubated with Sepharose 4B Conjugated 7-Methyl-GTP. The pulldowns were washed and analyzed for eIF4G, eIF4E, and 4E-BP associated with the cap, supernatants, or total cell lysates. (A) Representative western blot of m^7G-bound proteins and the total protein from cell lysates before pulldowns. (B) Fold changes in eIF4G and eIF4E following vehicle (0) or equol treatment at the m^7G cap relative to the total levels of eIF4G or eIF4E in cell lysate. N=3 ± SEM. An asterisk indicates statistical significance of $P\leq0.05$. (C) Relative IRES-dependent protein synthesis following equol treatment. MDA-MB-435 cells expressing a plasmid with a cap-dependent *Renilla* luciferase (RLuc) followed by a 5'UTR containing the p120 catenin IRES driving a firefly luciferase (FLuc) or control plasmid without an IRES, were treated with vehicle or equol for 24 h. Cells were lysed, and the relative IRES activity analyzed as 570 nm FLuc/480 nm RLuc. IRES activity was quantified relative to control activity for vehicle or equol treated cells. Results show fold change in IRES activity compared to vehicle for N=3 ± SEM. An asterisk indicates statistical significance of $P\leq0.05$. (D) A model of cap-independent (IRES) mRNA translation showing the potential effects of equol. Increased eIF4G expression by equol is expected to result in enhanced IRES-dependent mRNA translation, while eIF4E and

4E-BP remains at the m^7G cap. Poly A binding protein (PABP) interacts with the poly A tail of the mRNA and eIF4G. eIF4A, eIF4B, and eIF3 interact with eIF4G. eIF3 binds the scaffolding protein eIF4G and the 40S ribosomal subunit at the IRES.

Figure 1.

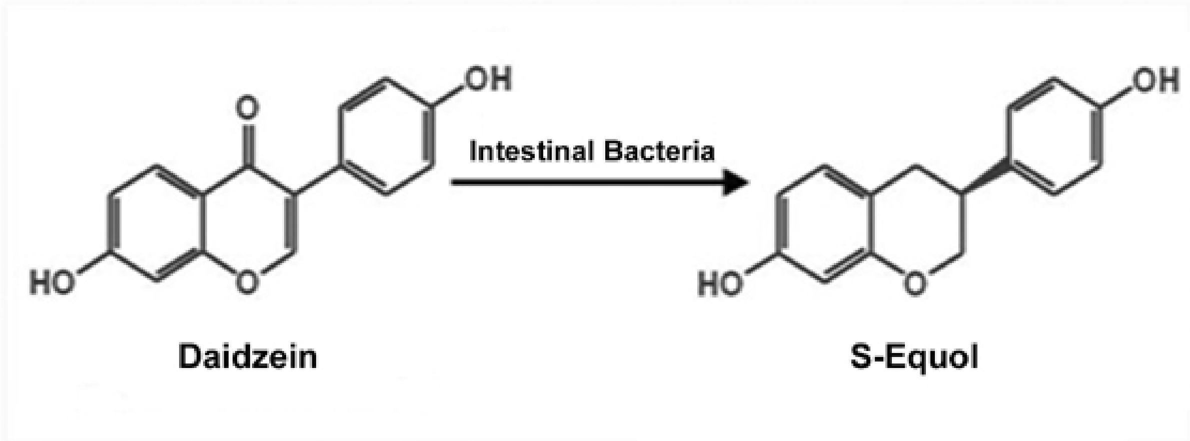
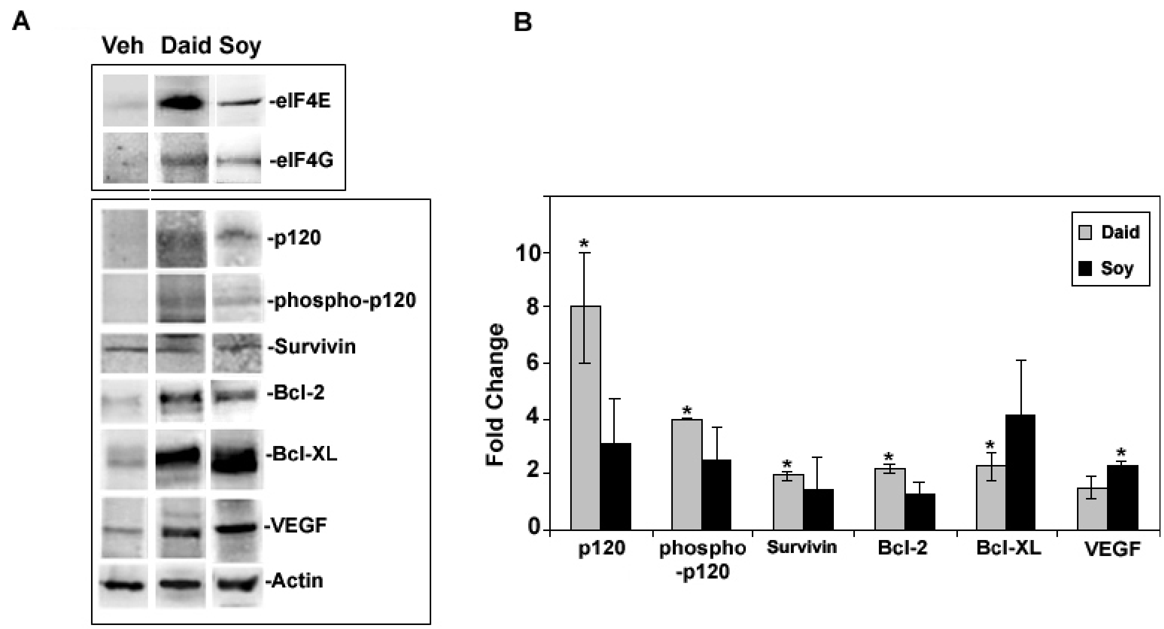
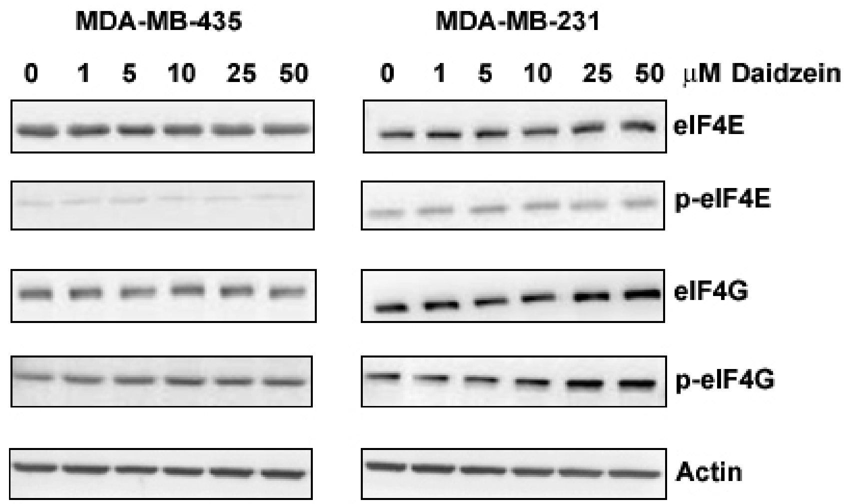


Figure 2.



A
Veh Daid Soy
-eIF4E
-eIF4G
-p120
-phospho-p120
-Survivin
-Bcl-2
-Bcl-XL
-VEGF
-Actin
B
Daid
Soy
Fold Change
10
8
6
4
2
0
p120
phospho -p120
Survivin
Bcl-2
Bcl-XL
VEGF

Figure 3.



MDA-MB-435
MDA-MB-231
0 1 5 10 25 50
0 1 5 10 25 50 μM Daidzein
eIF4E
p-eIF4E
eIF4G
p-eIF4G
Actin

Figure 4.

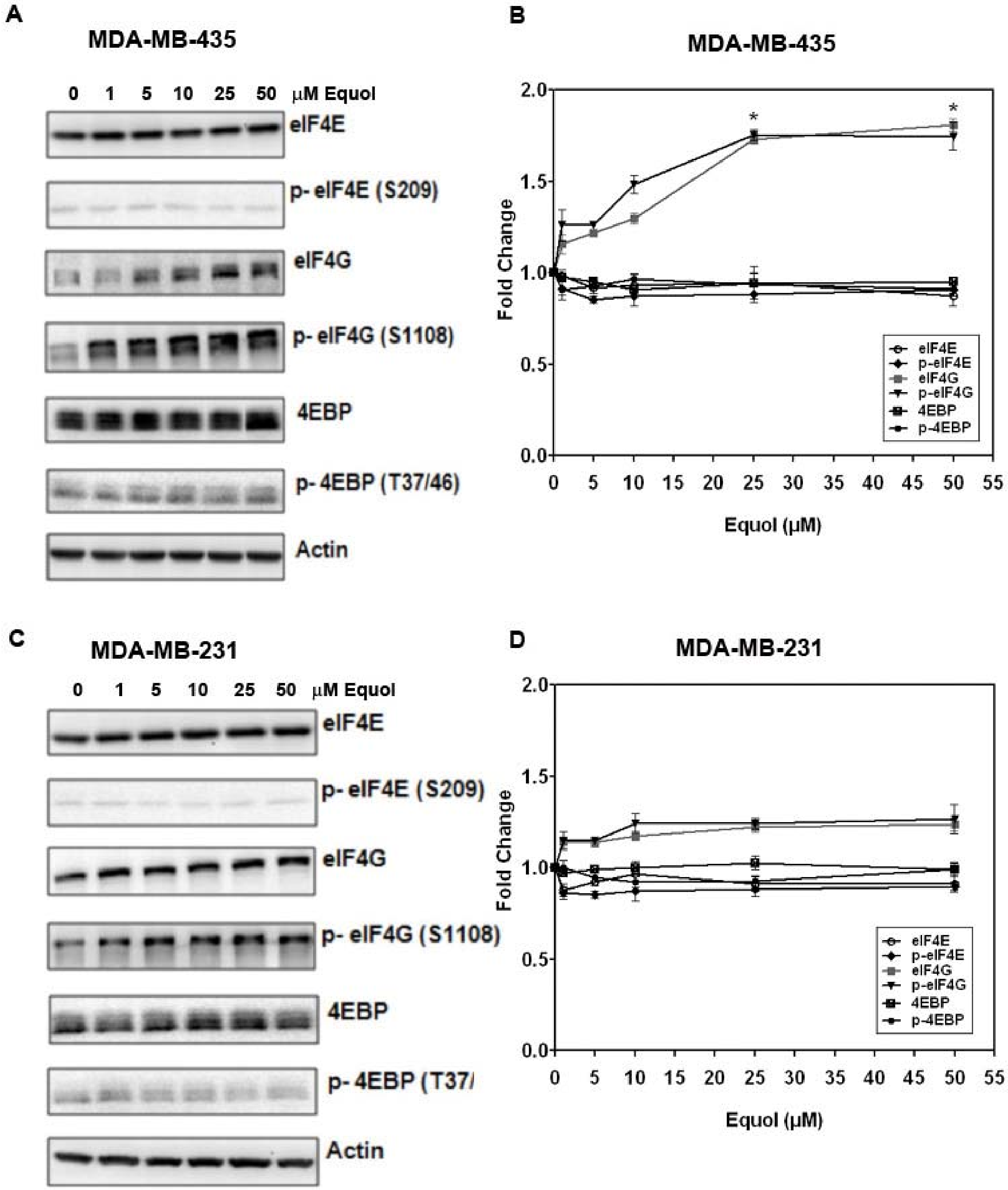

A
MDA-MB-435
0 1 5 10 25 50 µM Equol
eIF4E
p- eIF4E (S209)
eIF4G
p- eIF4G (S1108)
4EBP
p- 4EBP (T37/46)
Actin
B
MDA-MB-435
Fold Change
Equol (µM)
eIF4E
p-eIF4E
eIF4G
p-eIF4G
4EBP
p-4EBP
C
MDA-MB-231
0 1 5 10 25 50 µM Equol
eIF4E
p- eIF4E (S209)
eIF4G
p- eIF4G (S1108)
4EBP
p- 4EBP (T37/
Actin
D
MDA-MB-231
Fold Change
Equol (µM)
eIF4E
p-eIF4E
eIF4G
p-eIF4G
4EBP
p-4EBP

Figure 5.

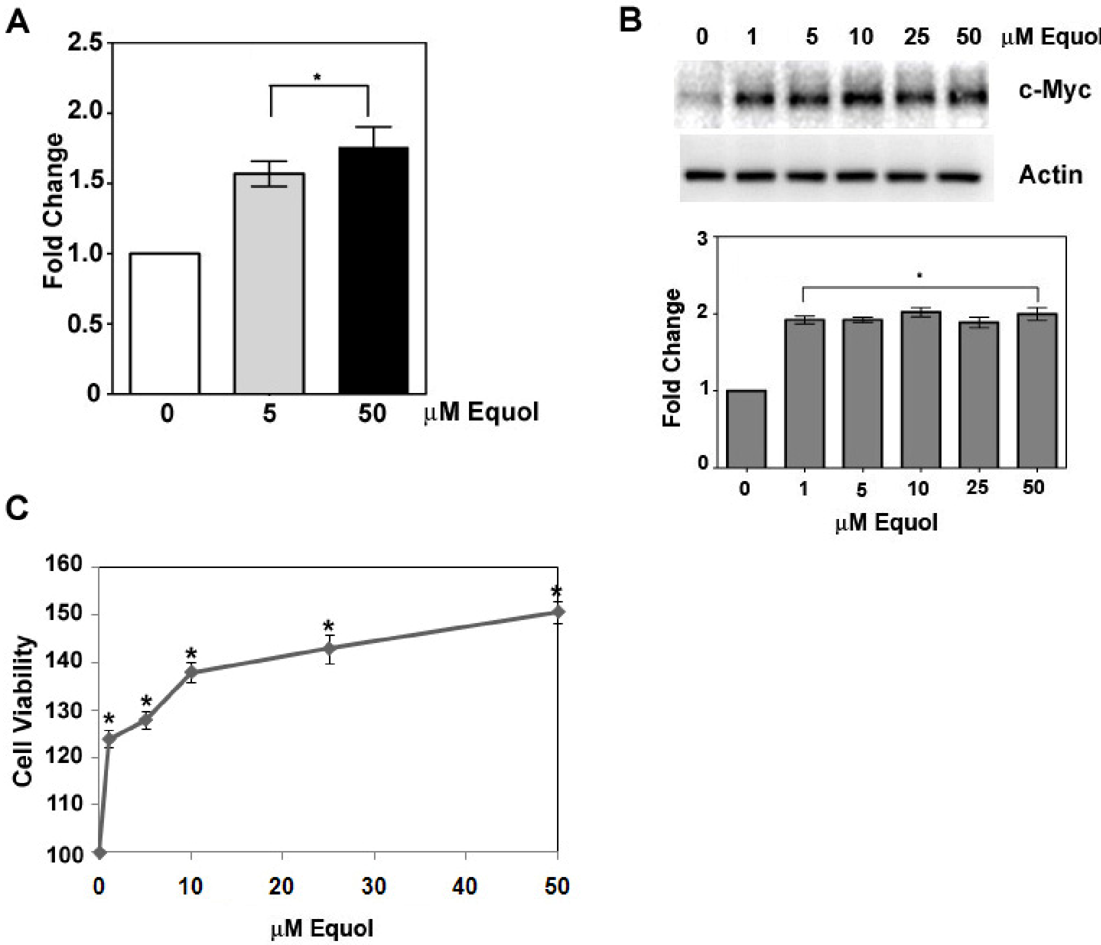

A
2.5
2.0
1.5
1.0
0.5
0
Fold Change
*
0
5
50
μM Equol
B
0
1
5
10
25
50
μM Equol
c-Myc
Actin
3
2
1
0
Fold Change
*
0
1
5
10
25
50
μM Equol
C
160
150
140
130
120
110
100
Cell Viability
0
10
20
30
40
50
μM Equol

Figure 6.

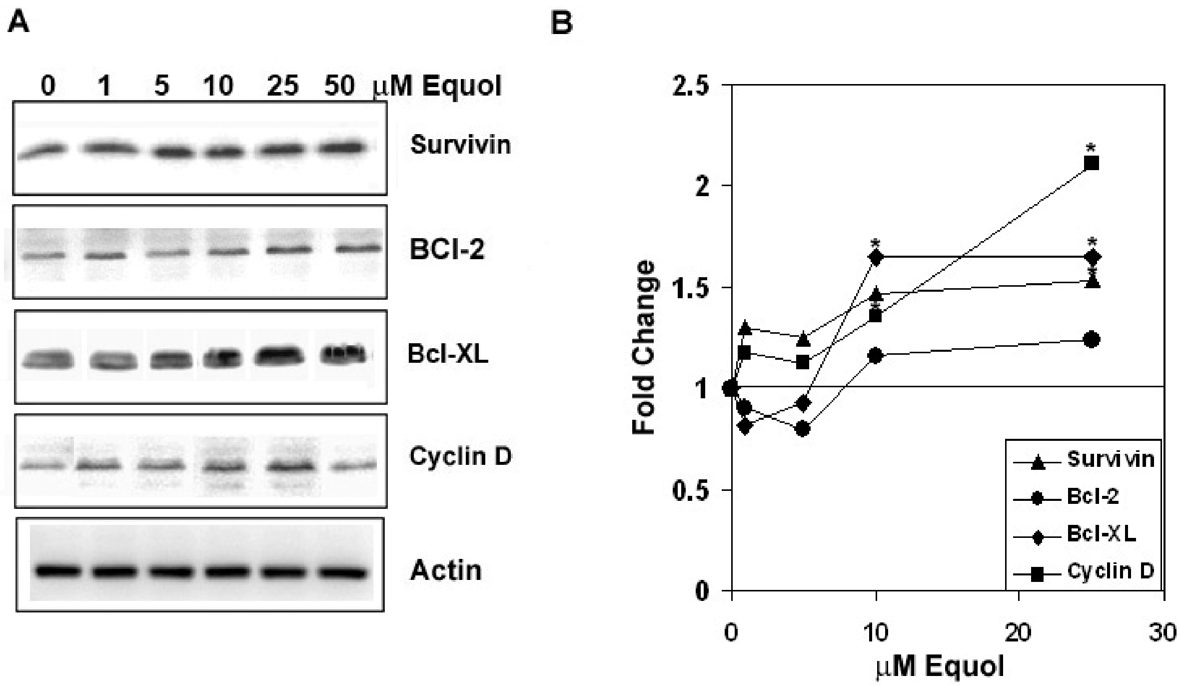
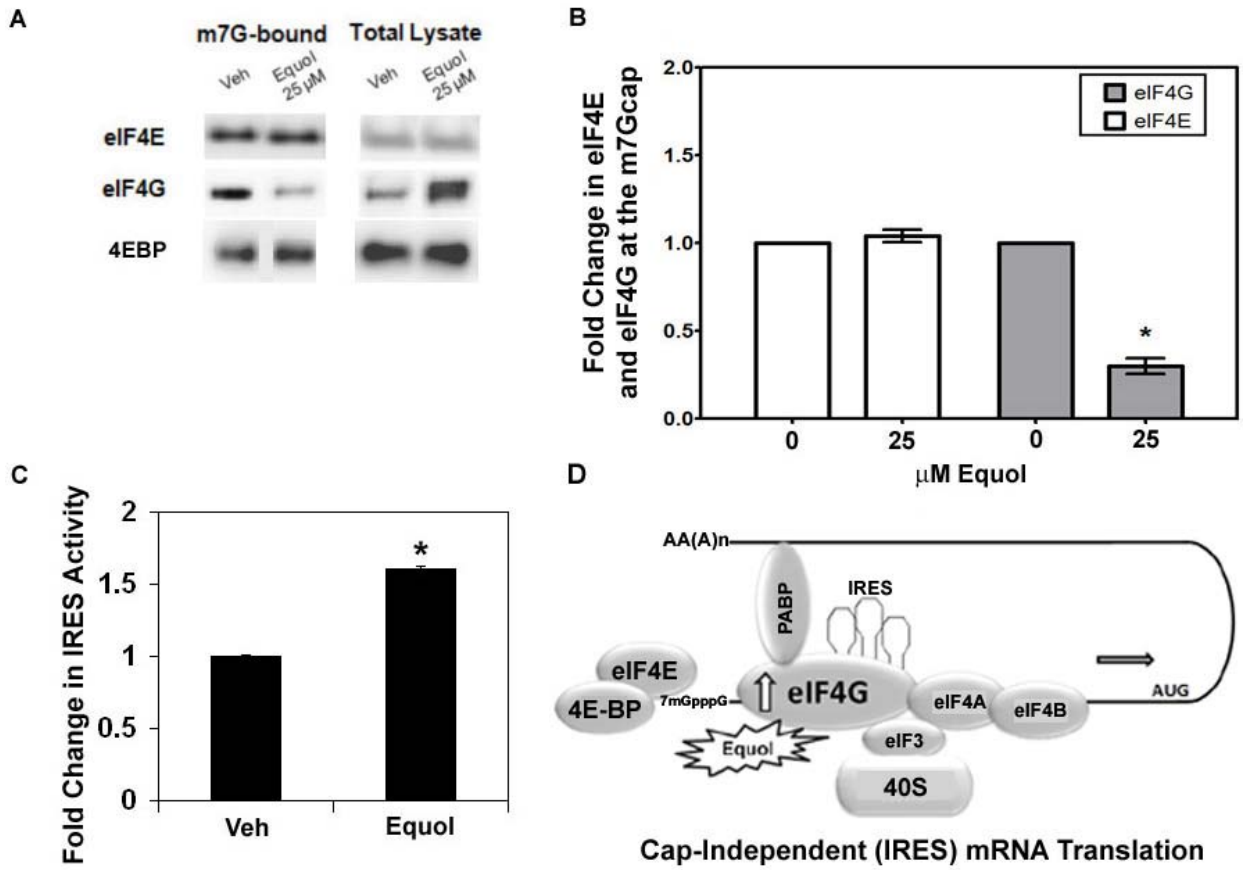

A
0 1 5 10 25 50 μM Equol
Survivin
BCl-2
Bcl-XL
Cyclin D
Actin
B
Fold Change
μM Equol
2.5
2
1.5
1
0.5
0
0
10
20
30
Survivin
Bcl-2
Bcl-XL
Cyclin D

Figure 7.


A
m7G-bound
Total Lysate
Veh
Equol 25 μM
eIF4E
eIF4G
4EBP
B
Fold Change in eIF4E and eIF4G at the m7Gcap
eIF4G
eIF4E
2.0
1.5
1.0
0.5
0.0
0
25
*
μM Equol
C
Fold Change in IRES Activity
2
1.5
1
0.5
0
*
Veh
Equol
D
AA(A)n
PABP
IRES
eIF4E
4E-BP
7mGpppG
eIF4G
eIF4A
eIF4B
AUG
Equol
eIF3
40S
Cap-Independent (IRES) mRNA Translation